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Abstract

Clinical trials in cystic fibrosis (CF) patients established proof-of-principle for transfer of the wild-type cystic fibrosis transmembrane conductance regulator (CFTR) gene to airway epithelial cells. However, the limited efficacy of gene transfer vectors as well as extra- and intracellular barriers have prevented the development of a gene therapy-based treatment for CF. Here, we review the use of new viral and nonviral gene therapy vectors, as well as human artificial chromosomes, to overcome barriers to successful CFTR expression. Pre-clinical studies will surely benefit from novel animal models, such as CF pigs and ferrets. Prenatal gene therapy is a potential alternative to gene transfer to fully developed lungs. However, unresolved issues, including the possibility of adverse effects on pre- and postnatal development, the risk of initiating oncogenic or degenerative processes and germ line transmission require further investigation. Finally, we discuss the therapeutic potential of stem cells for CF lung disease.

Keywords: Cystic fibrosis; CFTR; Human artificial chromosome; Animal model; Vector development; Gene delivery; *In utero* gene therapy; Stem cells

1. Introduction

Gene therapy is still far from becoming a viable curative treatment for cystic fibrosis (CF) patients. The currently achieved level of understanding and technological progress pose a number of interesting genetic, biological, pharmaceutical and regulatory problems. Only once these issues have been solved will gene therapy become a valid option for the treatment and cure of CF. This article is not intended to be a comprehensive review of the field, but rather a description of recent progress in the development of gene transfer vectors, preclinical animal models, *in utero* gene therapy and the application of stem cells as an experimental therapy for CF. We also include a discussion of long-term therapeutic strategies, which are currently in the pre-clinical stage of development, such as genomic context vectors and human artificial chromosomes based solely on the replication and segregation mechanisms of the host cell.

The cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in airway epithelia, on the luminal side of the plasma membrane, where it serves as a

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phosphorylation-regulated Cl^- channel and a regulator of channels and transporters [1–3]. In particular, activation of CFTR leads to parallel inhibition of the epithelial Na^+ channel (ENaC), which is lost when CFTR is absent or dysfunctional [2,3]. Current data support the "low volume" hypothesis of CF lung disease, which postulates that loss of Cl^- secretion and increased Na^+ absorption reduce the thickness of the airway surface liquid (ASL) overlying airway epithelia with the result that mucociliary clearance is impaired [4]. In addition, reduced CFTR-dependent bicarbonate secretion might affect the hydration of secreted mucus, affecting its physical properties [5]. CFTR is also expressed in submucosal glands in the airways, which play an important role in host defence. Loss of CFTR function in duct-lining serous cells prevents the secretion of mucus and anti-microbial factors by submucosal glands [6]. Together, these pathological features contribute to the formation of thick, dehydrated mucus, which provides an ideal environment for persistent bacterial growth, triggering chronic inflammation and ultimately, organ failure in the CF lung.

Although CFTR is widely expressed in epithelia lining ducts and tubes throughout the body, CFTR dysfunction in the lungs is the main cause of morbidity and mortality in CF patients. When the CFTR gene was cloned in 1989, CF appeared to represent an ideal disease to be cured by gene therapy, because it is a monogenic disease and the lungs are easily accessible. However, none of the clinical trials completed to date using either nonviral (cationic liposomes) or viral (adenoviruses and adeno-associated viruses) vectors achieved therapeutic correction of the basic defect and persistent wild-type CFTR expression in nasal and pulmonary epithelia of CF patients (for review, see [7–11] and Supplementary Tables 1 and 2, available online only). Nonetheless, these clinical trials provided proof-of-principle that CFTR cDNA-based expression cassettes can be transferred to airway epithelia, as evidenced by molecular (DNA or mRNA detection) or electrophysiological (nasal potential difference) techniques.

Gene therapy vectors have to overcome anatomical and cellular barriers prior to delivery of DNA to the relevant site, i.e. the nucleus of target cells. Most vector molecules delivered to the airway surfaces are rapidly lost by mucociliary clearance [12,13]. In CF airways, thickened mucus and mucus plugs further enhance the barrier to gene transfer. Additional barriers preventing efficient gene delivery to CF airway epithelia include the apical membrane glycocalyx, the lack of appropriate receptors at this location, and tight junctions between the cells. Submucosal glands that play an important role in CF lung disease are not accessible from the luminal side. Furthermore, we have to consider the histological and anatomical complexity of the lung. The proximal and distal airways are composed of a series of branching tubes lined by different epithelial cells that show distinct proximal to distal gradients in patterns of gene expression and function. Obviously, it is not trivial to emulate the normal pattern of CFTR expression with a relatively simple vector system.

2. Viral and nonviral vectors

Many viral and nonviral vectors have been tested for their usefulness in CF gene therapy. In this review, we will discuss vectors, which are currently used in the clinic or are in development for this purpose. This section will also present advances in the construction of DNA molecules designed to achieve physiological levels of CFTR expression.

2.1. Viral vectors

Adenoviral (AV) vectors are severely hampered by their low transduction efficiency of human airway epithelia and by their induction of strong immune responses [14]. In contrast adeno-associated viral (AAV) vectors are capable of long-term gene transfer and expression in bronchial epithelia of rabbits and nonhuman primates. Because AAV vectors are also devoid of strong inflammatory potential [15], they have been investigated as alternative viral vectors for CF gene therapy. Serotype 2 AAV vectors were tested in clinical trials with repeated administration to CF patients [16–18]. The main conclusions from these studies were that AAV2 vectors demonstrated some efficiency, but were limited by transient transgene expression due, at least in part, to the immune response to the vector. To avoid immune recognition and increase tranduction efficiency, AAV vectors with other serotypes have been constructed. The availability of AAV vectors in which a common AAV2-based genome is packaged in capsids from different AAV isolates demonstrates that AAV6 and AAV9 serotype capsids transduce airway epithelia at higher rates than the AAV2 capsid both *in vitro* and *in vivo* [19,20]. More recently, Limberis et al. [21] showed that AAV6 was most effective at transducing the mouse nasal epithelium *in vivo* and human ciliated airway epithelium *in vitro*, suggesting its potential for further preclinical and clinical testing. Because the cloning capacity of AAV vectors is too small for full-length CFTR expression cassettes, truncated CFTR variants have been developed [22]. In view of the complex interactions between CFTR and other cellular systems, it is uncertain whether this approach will complement all CFTR functions. A novel approach, which involves segmental trans-splicing between two AAV vectors expressing partial CFTR sequences [23], will require very high transduction rates to be effective *in vivo*.

In addition to the DNA viruses, AV and AAV, various RNA viruses have been validated for airway gene transfer. Murine parainfluenza virus type 1 [or Sendai virus (SeV)], human respiratory syncytial virus (RSV) and human parainfluenza virus type 3 (PIV3) all transduce efficiently airway epithelia cells by attaching to sialic acid and cholesterol [24,25], which are abundantly expressed on the apical surface of these cells. These viruses replicate in the cytoplasm and do not harbour a risk of insertional mutagenesis. Although RSV and PIV3 are human pathogens, SeV, the only RNA virus for which efficiency has been assessed *in vivo*, is not. Recombinant RSV and PIV3 vectors have been used to correct defective CFTR-mediated ion transport in primary cultures of human

Table 1
Comparison of viral vectors for CF gene therapy

Vector	Integration	Persistence of expression *in vivo*	Pro-inflammatory/ Immunogenicity	Biosafety and efficiency studies on animal models	Efficacy studies in cellular models (with CFTR transgene)	Efficacy studies on animal models (with CFTR transgene)	Clinical trials for CF
Adenovirus	No	No	High	Yes	Yes	Yes	Yes
Adeno-associated virus	Both episomal and integrated gene expression	Yes	Low	Yes	Yes	Yes	Yes
Sendai virus	No, cytoplasmatic	No	High	Yes	Yes	Yes	No
Parainfluenza virus	No, cytoplasmatic	Unknown	Human pathogen	No	Yes	No	No
Respiratory syncytial virus	No, cytoplasmatic	Unknown	Human pathogen	No	Yes	No	No
Lentivirus	Yes	Yes	Low	Yes	No	Yes	No
SV40	Yes	Yes	No	Yes	Yes	Yes	No

CF airway epithelia [26,27]. Of note, PIV3 restored ASL volume regulation and mucus transport to levels approaching those of non-CF ciliated airway epithelia [27] (Fig. 1). SeV-CFTR administration to the nasal epithelium of CF mice partially corrected defective Cl^- transport, albeit with a high degree of inflammation [28]. However, gene expression mediated by recombinant SeV-based vectors is transient and repeated administration does not seem feasible because of the development of neutralizing antibodies against vector [29,30].

Lentiviral (LV) vectors derived from human immunodeficiency virus type 1 (HIV-1) and feline immunodeficiency virus (FIV) are integrating retroviruses which can be adequately pseudotyped to achieve efficient transduction of airway epithelia [31,32] (Fig. 1). Treatment of nasal epithelia of CFTR knockout mice with LV vectors expressing CFTR generated sustained CFTR gene expression and corrected the electrophysiological defect for up to 12 months [33,34]. It is currently unclear, whether prolonged expression is the result of vector integration into pulmonary stem or progenitor cells or due to the long half-life of airway epithelial cells (up to 17 months according to recent studies [35]). Most importantly, LV vectors can be repeatedly administered to the airways of mice [36]. While this is very encouraging, it is vital that innate immune responses to LV vectors are thoroughly investigated. In one study a LV vector elicited a mild and transient induction of IFN-γ transcription in tracheal epithelial cells, whereas an AV-derived vector strongly activated NF-κB and different cytokine transcripts (e.g. ICAM-1, IL-8, RANTES, IP-10, TNF-α, IL-6, IL-1β [37]. These results argue that the "stealth" properties of LV vectors warrant further evaluation in preclinical animal models.

An important drawback of standard LV vectors pseudotyped with the vesicular stomatitis virus glycoprotein G (VSV-G) is their low efficacy of gene transfer to murine lung epithelia *in vivo*. Approaches suggested to increase the efficacy of gene transfer [14] include treatment of airway epithelia with lysolecithin [33], addition of viscous adjuvants [38] and tissue injury [39]. All of these strategies likely cause transient damage to airway epithelia, modification of the paracellular permeability, and expose previously inaccessible receptors.

An alternative approach to enhance gene transfer is to pseudotype LV vectors with heterologous envelope proteins that target the apical membrane of intact airways with high efficiency. Indeed, the envelope protein of the notorious Ebola virus provides an effective alternative to VSV-G [40, 41]. Other glycoproteins, including the baculovirus envelope protein GP64, have also been tested successfully in the airways [42–44]. The feasibility of LV-mediated gene transfer to fetal airway epithelium has also been examined because of the advantages of therapeutic intervention before disease development, the absence of a functional immune response and potentially the lower dose required [45–47] (see Section 4. "*In utero* gene therapy for cystic fibrosis?").

In a recent study, Mueller et al. [48] demonstrated that a novel minimal ('gutless') SV40 CFTR expression vector significantly reduced pathology in CFTR knockout mice challenged with *Pseudomonas aeruginosa*. The authors argue that SV40 is an attractive vector candidate because it offers stable expression through integration and is not immunogenic in rodents. Tests in larger animals and eventually humans are required to establish efficacy and safety of this vector system. For further information about the characteristics of viral vectors used in CF gene therapy, see Table 1 and Supplementary Table 2 (available online only).

2.2. Nonviral vectors

Among the many nonviral gene therapy vectors tested (see Supplementary Table 1), GL67 ([Cholest-5-en-3-ol(3b)-3-[(3-aminopropyl)[4-[(3-aminopropyl)amino]butyl] carbamate]) emerged as a promising lipid for efficient lung transfection based on structure-function studies [49]. However, two clinical trials produced contradictory results concerning its efficacy [50,51], most likely as a result of the different study protocols employed. Zabner et al [50] showed that naked DNA encoding CFTR was at least as effective as DNA-GL67 complexes at transfecting nasal epithelia. By contrast, Alton et al. [51] demonstrated that nebulising DNA-GL67 complexes into the lungs of CF patients led to a significant degree of correction of the Cl^- transport defect, whereas patients given the placebo (GL67 alone) showed none. Moreover, all patients

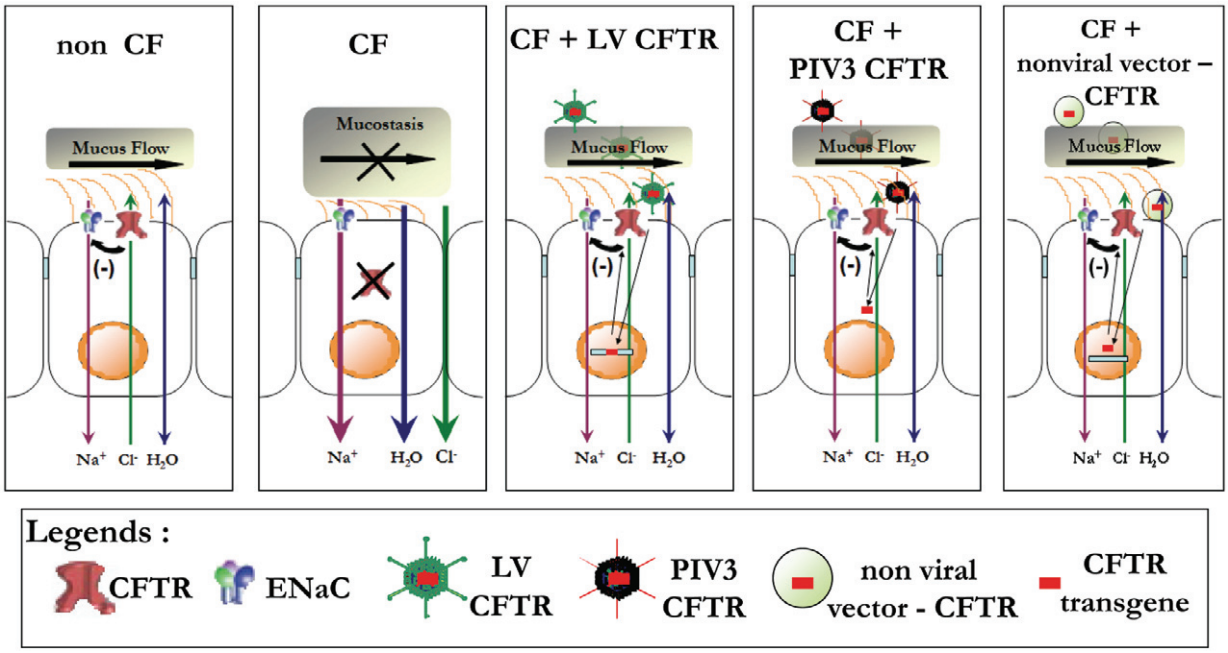

Fig. 1. CFTR gene transfer to the airway epithelium by viral and nonviral vectors and restoration of airway surface fluid (ASL) homeostasis. Schematic representation depicting the role of CFTR and ENaC in ASL homeostasis in non-CF airway epithelium; the presence of CFTR modulates ENaC activity and combined regulation of these ion channels dictates ASL depth regulation at a level sufficient for effective mucus transport (thick black arrow). In CF airway epithelium, the absence of CFTR reduces fluid secretion and leads to ENaC dysregulation resulting in hyperabsorption of surface fluid, dehydration of ASL, and mucostasis with accumulation of mucus plugs. Delivery of CFTR to CF ciliated cells by PIV, LV, or nonviral vectors should restore CFTR function, ENaC regulation, ASL homeostasis, and mucus transport. PIV is representative of viruses replicating in the cytosol, whereas lentiviruses are integrating vectors. Nonviral vectors transfect plasmid DNA which remains episomal in the nucleus. The nonviral delivery of mRNA could be an alternative to plasmid DNA, avoiding the nuclear membrane barrier (not shown).

showed mild flu-like symptoms for a few hours immediately after nebulization [51]. This unfavourable outcome was attributed to unmethylated CpG dinucleotide motifs present in bacterial DNA. Consistent with this idea, a CpG-free plasmid, complexed with GL67, directed sustained *in vivo* expression of CFTR mRNA for at least 56 days after aerosol delivery to the mouse lung without eliciting an inflammatory response [52]. This study also demonstrated that the duration of CFTR gene expression, which usually lasts for 1–4 weeks, can be extended by substituting a human housekeeping promoter for the commonly used viral promoters. Using the ubiquitin C promoter, the duration of CFTR expression was extended to 6 months or more after a single administration to the murine lung [52,53].

GL67 is the delivery system of choice for the forthcoming nationwide CF gene therapy clinical trials in the UK in a larger cohort of patients [54]. The key question is whether the level of gene transfer mediated by GL67 is sufficient to improve clinical parameters. To address this issue, clinical trials will need to be performed for long enough to have a realistic chance of altering the underlying pathophysiology (thus requiring repeated administration) using improved preclinical tests and clinically relevant endpoints to measure efficacy [55,56] (Fig. 1).

The use of messenger RNA (mRNA)-based nonviral gene transfer is a new strategy to express CFTR in target cells [57]. By choosing mRNA instead of plasmid DNA as the transgene, transfection efficiency depends solely on the cytoplasmic expression machinery. A key advantage of this approach is that the nuclear envelope, one of the major obstacles to nonviral gene therapy, does not have to be overcome. Messenger RNA transfection protocols are similar to those for plasmid DNA and simpler than those for viral transduction. In the past, mRNA-based gene therapy strategies were limited by the cost of producing large amounts of translatable mRNA. However, now mRNA generated by *in vitro* transcription from plasmid templates can be obtained from commercial sources at affordable prices. There have also been concerns about the stability of mRNA and handling difficulties. However, several papers have recently demonstrated that mRNA can be successfully used to express transgenes in mammalian cells and tissues [58–61]. Of note, mRNA transfection results in controllable, transient transgene expression. Moreover, this method does not modify the genome, avoiding the risk of insertional mutagenesis.

When compared to DNA, much less is known about immune responses to RNA, although responses to both seem to be mediated by Toll-like receptors (TLR). Interestingly, innate immune recognition of RNA by TLR3, TLR7 and TLR8 appears to be controlled by nucleotide modifications, including methylation (see http://medlib.med.utah.edu/RNAmods). Because, *in vitro* transcribed RNA elicits robust immune responses by cultured dendritic cells [62], it will be critical to understand better these "protective" *in vivo* nucleotide modifications. Finally, mRNA-mediated gene expression diminishes rapidly (i.e. <3 days) *in vitro* and *in vivo* [61]. Future research

should address this drawback, which limits significantly the therapeutic potential of mRNA-mediated transfection.

2.3. Genomic context vectors for delivery of therapeutic CFTR locus

Effective therapy by *in vivo* delivered therapeutic DNA requires physiological levels of gene expression maintained for prolonged time periods. To achieve this goal, the use of genomic fragments that contain all the long-range control elements allowing tissue-specific gene expression at physiological levels has been proposed [63]. Such a goal requires knowledge of the critical regulatory elements in the CFTR locus. In the following section, we present an up-to-date review of those elements and discuss the progress made in the field of genomic context vectors (GCVs).

2.3.1. The CFTR locus: genomic and functional analysis

The *CFTR* gene maps at 7q31.2 and has a primary transcript of 189 kb. Its expression is regulated temporally during development and spatially in different tissues. The *CFTR* locus is flanked by genes with different tissue-specific expression profiles, suggesting the presence of specific control elements and insulators (Fig. 2). Nuclear localization studies of *CFTR* and its adjacent gene loci in humans and mice demonstrate that different chromatin regions behave independently, depending on their expression profiles [64,65]. This implies the presence of a finite chromatin block between adjacent genes in the *CFTR* locus [64,65]. Importantly, these studies also revealed that the patterns of nuclear localization are not conserved between human and mouse [64,65].

In 1991, Anand et al. [66] isolated the entire human *CFTR* locus in a 320 kb yeast artificial chromosome (YAC) vector (YAC 37AB12). Subsequently, YAC 37AB12 was used to complement the loss of CFTR function in CFTR knockout mice [67]. Accordingly, YAC 37AB12 achieved physiological levels of human CFTR expression in many of the murine cells in which *CFTR* is normally expressed, demonstrating that the human protein effectively restores CFTR function in CFTR knockout mice [67]. However, the molecular mechanisms by which YAC 37AB12 regulated tissue and temporal specific *CFTR* expression were not elucidated. Analysis of distinct promoter regions from CFTR homologues in different species failed to reveal a conserved structure of promoter elements and did not detect the presence of tissue-specific promoters as evidenced by the absence of TATA elements [68–70]. These studies suggest that CFTR expression is regulated by other mechanisms. Harris and colleagues mapped DNaseI hypersensitive sites (DHS) along the entire *CFTR* locus with high-resolution methods and identified a number of cell-type specific DHSs within and flanking the gene (for review, see [71]) (Fig. 2). Two DHSs were mapped at −20.5 and −79.5 kb from the transcription start site. A cluster of five DHSs was identified in the region 3' to the CFTR gene at +5.5, +6.8, +7, +7.4 and +15.6 kb and eleven DHS clusters were identified within introns 1, 2, 3, 10, 16, 17a, 18, 20 and 21 [71]. Although these DHSs did not fully correlate with cell-type specific CFTR expression, the intron 1 DHS corresponds to a regulatory element that specifically controls CFTR promoter activity in intestinal cells [72,73]. Accordingly, removal of this element from the CFTR-YAC caused a reduction of CFTR expression by 60% in transgenic mice, but only in the small intestine [74]. More recently, Blackledge et al. [75] demonstrated that the +6.8 DHS functions as an insulator and mediates, in cells that express CFTR, interaction with the *CFTR* promoter. This suggests that the *CFTR* locus exists as a loop, which is characteristic of an active chromatin hub [75] (Fig. 2). Finally, it was suggested that suppression of CFTR transcription does not involve promoter methylation [76].

2.3.2. Human artificial chromosomes for CFTR expression at physiological level and long-term maintenance

The principal aim of gene therapy in CF is to develop a DNA molecule conferring cell-type specific and physiological CFTR expression and possibly high mitotic stability. The complete genomic *CFTR* locus, including regulatory elements, is expected to provide the natural genomic context, similar to that found on endogenous chromosomes, thus allowing expression of the correct protein at the right time in the various stages of regenerating and differentiating cells. In principle, cell-type specific vectors would not require tissue-specific delivery since cells normally not expressing CFTR would also not express the therapeutic gene. Applied through stem cells, the naturally-regulated gene could also correct different cell types participating in organ regeneration. A feasible approach would be the assembly of the therapeutic artificial chromosome *in vitro* and transfer into stem or iPS (induced pluripotent stem) cells that can subsequently be used to colonize the target tissues/organs upon implantation [77]. This approach might also include the use of tracking markers that allow visualization and isolation of progenitor cells using the microRNA machinery [78].

Effective endogenous replicators combined with a centromeric function in the vector are expected to substantially improve stability of expression after delivery. Clearly, the development of such regulated and spontaneously replicating vectors is a challenging feat of genetic engineering. It requires a thorough understanding of chromosomal elements that govern transcription regulation and replication. A decade of research on human artificial chromosomes (HACs) has shown that several members of alpha satellite DNA sequences, the principle class of tandemly repeated DNA of human centromeres, efficiently formed centromeres *de novo* [79–81]. Naked DNA arrays >80 kb transferred into the human lung sarcoma cell line HT1080 efficiently formed distinct chromatin regions of a functional centromere and bound the centromere-specific histon H3 variant CenpA (centromere protein A) indicative of active centromeres. Shorter regions were less efficient and the newly formed centromeric chromatin extended into adjacent, non-centromeric input sequences, potentially interfering with gene expression. Isogenic alpha satellite sequences below ∼30 kb completely lost their seeding activity [82–84], suggesting that functional regions cannot efficiently assemble from short input molecules *in*

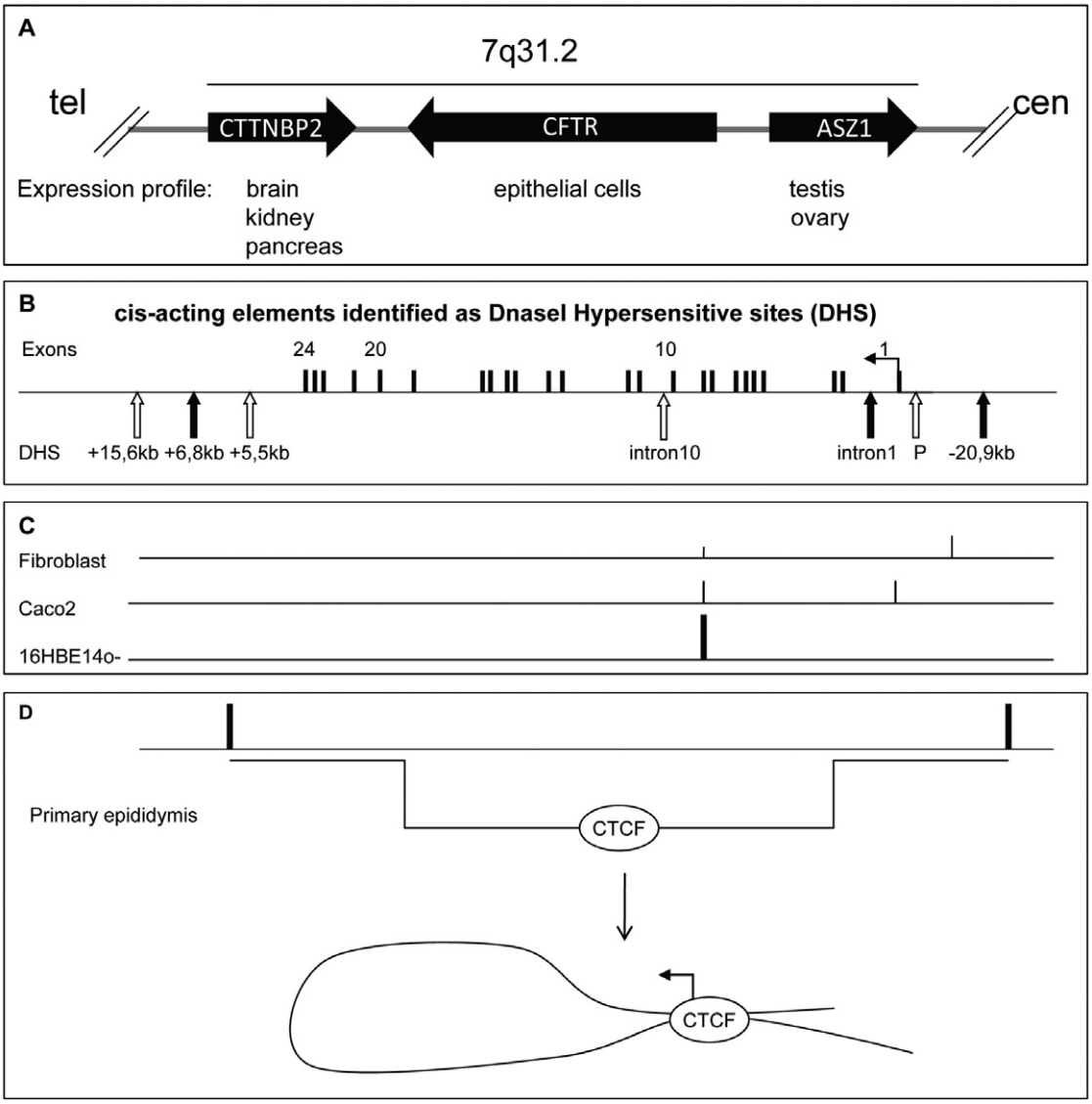

Fig. 2. Cis-acting elements possibly involved in tissue-specific expression of the CFTR gene. (A) the CFTR locus maps at position 7q31.2 and is flanked upstream by ASZ1 and downstream by CTTNBP2; these three genes have different expression profiles, as indicated. (B) schematic representation of the CFTR locus; the exons are indicated by vertical bars; DNaseI hypersensitive sites (DHSs) are indicated by arrows; black arrows identify sites involved in tissue-specific expression (intron 1 DHS positively regulates promoter activity in intestinal cells, −20.9 kb and +6.8 kb are bound by the transcriptional repressor CTCF (CCCTC-binding factor) in primary epididymis cells). (C) DHSs mapped by a high-resolution method in cells that do not express (skin fibroblasts) or express (Caco2 and 16HBE14o−) CFTR. DHSs are indicated by vertical bars, height and thickness of the bars are proportional to the intensity of the DHS signal. Of interest is the DHS in intron 10, which was found in the bronchial epithelial cell line 16HBE14o−, but not in the other cell lines. Further investigation is required to assign a role to this element. (D) in primary epididymis cells −20.9 kb and +6.8 kb DHSs, previously identified as enhancer-blocking insulators, bind CTCF, which promotes formation of a chromatin loop (chromatin hub).

vivo. It has also been established that genomic genes can be stably expressed in the vicinity of *de novo* centromeres [65,85,86].

Since the assembly of the first HACs, which were obtained using either uncloned tandem repeat arrays or arrays cloned in a YAC [79,80] and since the first *CFTR* locus inserted into a pre-existing human minichromosome [66,87], PACs (P1 artificial chromosomes)/BACs (bacterial artificial chromosomes) have become feasible cloning tools capable of solving the principal problems of scale up, storage, and DNA damage. So far different prokaryotic artificial chromosome vectors are available, including: (i) PAC/BAC clones covering the entire *CFTR* locus and for which the CFTR exons sequences are available [88]; (ii) one vector based on the Epstein-Barr virus (EBV) replication mechanism [89] and (iii) BAC vectors containing the entire CFTR locus plus additional up- and down-stream sequences cloned by a single step procedure [90]. Some of these vectors have been shown to drive production of functional CFTR in model cells [89,90]. Furthermore, novel methods have been devised, including PAC cloning and long PCR of 20–60 kb with a very low error rate to tailor loci and boundaries, and delivery mediated by invasive bacterial cells. A PAC vector of 159 kb containing the *CFTR* locus from approximately −60 kb 5′ to the middle of intron 9 to

which a synthetic CFTR-EGFP fusion was added, showed expression and correct splicing in HT1080 when the vector was delivered either by bacterial cells (bactofection) or by lipids (lipofection) [91]. Ongoing research applies re-constructed versions of the *CFTR* locus on HACs, either based on large wild-type sequences, engineered loci with variants for an optimized protein, or silent variants easing the RT-PCR based discrimination between the transgene and endogenous *CFTR* genes.

The final size and exact arrangement of the functional elements on a therapeutic *CFTR*-HAC are still unclear. Further, the analytical power has to be improved for routine *de novo* HAC detection in small samples of primary cells and for fine structural and functional analyses. Animal transgenics with *de novo* HACs and the development of transfer methods early in life using suitable animal models (see Section 3. "Animal models old and new") need to be developed to study delivery, mitotic persistence, and test for meiotic incompetence.

In principle, HAC construction allows the exclusion of virtually all non-human vector portions, and since HACs are based on chromosomal elements of human origin they should be well tolerated and should not elicit undesired immune responses such as those induced by bacterial CpG-rich sequences [92,93]. To date the most reliable technique that allows therapeutic use of HACs is the combination of patient-specific iPS or stem cells and HACs assembled *in vitro* with the normal version of the defective gene. Proof of principle has recently been established using Duchenne muscular dystrophy (MDM) as a model disease and an HAC containing the dystrophin locus (DYS-HAC). Indeed, DYS-HAC-mediated correction of MDM was obtained in the mdx mouse model and in patient-derived iPS cells [77]. Nonetheless, it is also reasonable to predict that in future the availability of a detailed functional map of the CFTR locus and a better definition of the DNAs required to assemble chimeric vectors that retain elements important for tight regulation and replication of the gene in different tissues will allow direct assembly of therapeutic HACs in target cells.

3. Animal models old and new

3.1. Mouse models

Mice, including strains carrying *Cftr* mutations have been widely used to test the efficacy of CFTR expression vectors. The primary target of these vectors is the airway epithelium, in view of its clinical prominence in CF pathology. The histology, physiology and phenotypic characteristics of normal and CF mutant lungs and airways are reviewed elsewhere in this issue [94]. To evaluate the results of gene transfer experiments and their relevance for CF gene therapy, it is important to understand the complex cellular design of the mouse airways and differences with the human lung. The mouse nasal cavity is lined in part by a specialized olfactory epithelium, mainly sustentacular cells, and with airway epithelium consisting mainly of ciliated and mucus producing non-ciliated cells. Numerous submucosal glands produce fluid and mucus. Because of its apparent similarities with human bronchial airways, this tissue is considered most relevant for CF gene therapy studies. The mouse tracheal epithelium resembles the human nasal airway epithelium with respect to cellular composition, but gene expression gradients are observed. In the lung, the mouse airways are covered with ciliated and Clara cells, resembling distal bronchioles in human. CFTR is expressed in surface epithelial cells and submucosal glands as was shown by electrophysiological characterization and in situ hybridization ([95]; for review, see [94]). It should be noted that the distribution of submucosal glands in murine airways is significantly different from the human pattern; mouse glands are restricted to the proximal region of the trachea and are absent from bronchi. Moreover, inbred strain background and CFTR genotype influence the murine distribution of submucosal glands [96,97]. The importance of these differences in relation to the relevance of mouse models of CF lung disease is unclear. Immunohistochemistry studies of mouse CFTR expression are limited by the properties of the available antibodies, but confirm proximal to distal mRNA gradients of CFTR expression and further suggest a distinct cell-specific pattern.

Current gene transfer vectors delivered by intranasal or intra-tracheal application hit cells at random and depending on their nature, create a CFTR expression pattern that does not necessarily match that of the endogenous gene. In this context almost every available vector system has been tested and reviewed (see Section 2. "Viral and nonviral vectors" and Supplementary Tables 1 and 2, available online only). Most of these studies concentrate on the efficiency of delivery, while some studies show a partial correction of the electrophysiological abnormalities in CF epithelia [98–100]. Only few studies so far report a significant change in a CF related phenotypic trait. Nonviral systems including plasmid/liposome formulations are effective in transfecting the nasal epithelium, but high transfection rates and functional complementation of the CFTR defect in mutant mice are difficult to achieve [101]. Cationic polymers like simple polyethylenimine (PEI) are effective delivery systems in the mouse lung, but their toxicity is a concern [102], although when formulated with serum albumin their efficiency is not diminished by CF sputum [103]. Advanced polyplexes have been developed and tested in a mouse model to find a delivery system with improved qualities [104]. Both AV [105,106] and AAV vectors [21] efficiently transduce murine lung and nasal epithelia. A significant effect on susceptibility to fungal infection was reported in a CF mutant mouse model using an AAV vector expressing a truncated CFTR [107]. The use of LV vectors and SV40 derived vectors in mouse models was discussed above (see, Section 2.1. "Viral vectors").

While these studies give a good and relatively affordable impression of the principal capabilities of vector systems *in vivo*, several concerns have to be addressed. First, the size and architecture of the human airways are substantially different from those of the mouse. Therefore, vector delivery has to be studied in large animals and eventually in clinical trials. Concerning phenotypic complementation, no animal models other

than CF mutant mice were available until very recently. The absence of pathology in the mouse nasal airways and lungs that can be quantified easily, together with the limited efficiency and stability of current delivery systems does not allow a reliable prediction of the therapeutic capabilities in human.

3.2. Large animal models

Set against its many advantages, the major drawback of the mouse as a model for pulmonary gene therapy is its small size. Mice have approximately 10^6 airway epithelial cells, while humans have 10^4 times that number [108,109]. Larger animal models for assessing gene delivery with null *CFTR* have therefore been developed in pig and ferret. Sheep, whose lungs are comparable in size and physiology to human lungs, have been extensively used in recent years for *in vivo* testing of pulmonary gene delivery. Although the sheep has normal ovine CFTR activity, the model nevertheless enables the efficacy of human CFTR gene transfer to be assessed at the RNA and protein level, and also enables any toxic effects of human-equivalent doses to be measured [110,111].

Through gene-targeting, Rogers et al. [112] succeeded in generating *CFTR*-knockout pigs which are all born with severe meconium ileus requiring surgical intervention [113]. While nasal electrophysiology confirms the lack of functional CFTR in CFTR knockout piglets, early indications are that their lungs and airways are histopathologically normal at birth. CFTR−/F508del piglets already exist and appear to have a similar phenotype to CFTR knockout (−/−) littermates [114]. We eagerly await the development of CFTR F508del/F508del animals. However, the data of Ostedgaard et al. [115] and Liu et al. [116] suggest that porcine F508del-CFTR might not be as severely misprocessed as its human equivalent, suggesting that a milder CF phenotype might be expected in the F508del/F508del pig model. It remains to be seen whether homozygous CFTR mutated pigs will go on to develop spontaneous infections and CF-like lung disease. However, recently a limited number of long-lived (3 month) CFTR−/− and CFTR−/F508del animals were diagnosed with airway wall thickening, increased bacterial load, inflammation and mucus plugging [117].

Great progress towards a ferret CFTR knockout model has also been made [118]. Its human-like airway morphology and cell types make the ferret a compelling model for studying lung disease, though it suffers from the scale problem in terms of its usefulness as a surrogate for humans in assessing dose-equivalent nebulised gene delivery. On the other hand, this also implies that experiments with this model are relatively affordable. Furthermore, the ferret is routinely used in viral lung infection studies [119], which implies that a large body of data and expertise is available. Current reports show that similar to the pig and mouse models, intestinal disease causing severe perinatal mortality is the dominant trait of the CF ferret [120]. Therefore, further attempts are aimed at obtaining a "gut corrected" strain expressing normal CFTR in intestinal epithelia only, which may develop lung disease in the absence of intestinal complications [120].

If progressive lung disease can be confirmed and methods are developed to extend the lifespan of the mutant animals, both the pig and the ferret model would become useful models to study vector delivery to CF airways.

4. *In utero* gene therapy for cystic fibrosis?

None of the gene therapy approaches for CF presently under development has yet reached effective clinical application. So far all clinical gene therapy studies for CF were applied to adolescent or adult patients in whom disease manifestation have already caused irreversible tissue damage. Some presently planned clinical trials indicate a trend towards application at earlier ages [121] and this trend would be most consequently pursued by *in utero* (fetal, prenatal) gene therapy.

The underlying hypotheses of *in utero* gene therapy are (i) that it might allow targeting of tissues/organs, which are inaccessible later in life before early onset tissue damage occurs, (ii) that it might achieve permanent therapeutic transgene expression in expanding stem cell populations using integrating vectors and (iii) that it might induce tolerance to vector and transgenic protein. The small size of the fetus also provides an advantageous vector to cell ratio, which is relevant for considerations of both efficiency and vector production costs. Successful fetal gene therapy could therefore offer prenatal prevention of disease, particularly in early manifestation conditions, provide a third option in addition to acceptance of an affected child or abortion, and broaden the scope and change attitudes towards prenatal screening.

Experimental work on rodents, sheep and non-human primates over the last 15 years has indeed verified the above hypotheses: In rodents, work demonstrated the feasibility of effective *in utero* transgene delivery to and expression in virtually all tissues and organs relevant for various genetic diseases [122]. Evidence for development of postnatal tolerance against the expressed transgenic protein has been provided [123,124] and evidence for stem cell transduction and clonal expansion of transduced cells also shown [125]. Collaborative work with Fetal Medicine specialists has shown that obstetric technologies, as used for the treatment of human fetuses, can be applied for efficient gene delivery to large animal models representative of the human fetus [45,126]. Finally, first proof of principle for curative *in utero* gene therapy for a few human genetic conditions have been provided in animal models of these diseases including conditions of the eye, brain, liver, muscle and the blood clotting system [127].

Given these results and the ongoing rapid progress in the development of more efficient, tissue specific and safer vector systems, an application to CF appears to be entirely feasible. This would, however, need a dedicated effort, preferably on a relevant large animal model; if possible one for CF. Effective marker gene expression in the fetal airways was shown using AV, pseudotyped LV and AAV vectors [21,41,128,129]. The AV vector was also used on the sheep fetal model to demonstrate efficient vector application by minimally invasive transcutaneous ultrasound guided intervention [129]. Further

work is now needed to develop novel vector systems for efficient gene transfer to airway epithelia, mediating longer-term expression of marker genes and easily detectable CFTR. Such studies would not only be relevant to CF, but also to other genetic diseases of the respiratory tract, such as surfactant B deficiency and alpha-1 antitrypsin deficiency. New generations of safe LV or AAV vectors appear presently as the most hopeful vectors for this task. In particular, safety issues surrounding vector-associated oncogenesis, which has been shown for onco-retroviral and LV vectors [130,131], and for which there is also some evidence with AAV vectors [132], are highly important. Oncogenicity is a general risk factor for gene therapy when vector systems with the potential for integration into the host genome are applied. In fetal life this may be particularly relevant since many genes, which we commonly know as oncogenes are physiologically active in cell proliferation and differentiation during early development and may therefore be more prone to vector insertion and hence, dysfunction at this time rather than later in life.

Other more hypothetical risks of *in utero* gene therapy, such as germ-line transfer, and toxicity of the transgenic protein also deserve consideration. Germ-line transfer would most likely depend on vector type and application route. Since the germ cells are well compartmentalized at the gestation time proposed for *in utero* gene therapy, the vascular or intra-peritoneal routes would be the most likely ones to carry such risk. This is no different from the presumed risk in postnatal gene therapy. Low-level retroviral transduction of germ cell progenitors was detected in male or female gonads after intra-peritoneal vector application to fetal sheep and monkeys, respectively [133,134]. However, no evidence of germ line transmission could be found in rodents or sheep after fetal vector administration in experiments designed to address this question [135]. For both pre- and postnatal gene therapy, this problem requires monitoring in the context of any clinical trial. Important consideration should be given to the risk of untimely, ectopic or over-expression of a transgenic protein *in utero* causing adverse effects on fetal and postnatal development [136]. This will be almost impossible to predict, would be specific for individual proteins and would most likely also depend on the gestation age at vector application [137]. Stringent control of expression to make sure that the protein is expressed only in the target cells and at physiological levels should minimize such risk. With regard to CF, it is of interest to note that the expression of CFTR in the lungs is at its highest levels in mid-gestation [138]. Animal experiments may not be entirely reliable in predicting outcome in a human fetus and, therefore, careful prenatal monitoring will be required and life-long postnatal observation of any *in utero* treated individual should be conducted as in any other gene therapy trial.

The novelty of fetal gene therapy demands particular ethical consideration of risks, benefits and alternatives. *In utero* gene therapy requires an exact prenatal diagnosis. At this stage, it is too late for *in vitro* fertilization-preimplantation selection, which requires preconception genetic analysis. The options after prenatal diagnosis of a genetically affected fetus are acceptance or termination on medical grounds. In CF, acceptance of an affected child and hope for a postnatal curative therapy or at least for further improvements of symptomatic therapies are well founded as would be a decision for termination to avoid the unknowns of the individual disease manifestation, prognosis and therapy. Although the benefits of successful *in utero* gene therapy would be very high compared to the former options, the risk of failure is very difficult to assess in an introductory phase. It is therefore not likely that CF would rank high for initial *in utero* gene therapy trials.

The situation might be very different in severe genetic diseases for which no therapy is available and particularly for families objecting in principle to abortion. Such conditions would most likely be the first targets of an *in utero* approach. On the basis of solid preclinical evidence for a good chance of a curative outcome and with full information of the possible risks, such families might wish to participate in a clinical trial of *in utero* gene therapy. If proven successful with such conditions and depending on the progress of alternative therapies, other diseases, including CF, could also be considered. However, quite obviously more work on vectors and animal models is still needed to reach a level of scientific confidence for potential success that would justify a human clinical application of *in utero* gene therapy for CF.

5. Promises and pitfalls of stem cell-based therapy

The concept is emerging that CFTR dysfunction may directly affect regulatory activities of cells involved in immune-surveillance and host response. It has been reported that polymorphonuclear leukocytes (PMN) express functional CFTR protein, which regulates phagolysosomal function [139]. Moreover, several lines of evidence suggest a genetic component to altered PMN function in CF [140]. In alveolar macrophages, CFTR appears to control phagosome acidification and bacterial killing, [94,141]. Accumulating evidence indicates that platelets, in addition to their well-known haemostatic functions, may play a role in inflammation and its resolution [142]. CF platelets display an array of defects, including the generation of lipoxins, well-known mediators of inflammation resolution [143].

Given the high PMN burden and inflammatory response in the CF lung, anti-inflammatory therapy has been suggested for treatment of CF lung disease. Both corticosteroids and non-steroidal anti-inflammatory drugs have been used in this context with mixed results [144,145]. The finding of abnormalities in CF in circulating and resident immune cells suggests the possibility of hematopoietic stem and progenitor cells (HSPCs) as a new target for CF gene therapy.

Stem cells are cells that have the capacity for unlimited self-renewal, meaning that they divide asymmetrically, both renewing themselves and producing a more differentiated daughter cell. This property has elicited excitement as to the use of these cells for therapeutic application. Indeed, HSPCs have been widely used in the clinic in recent decades for allogeneic transplantation of patients with hematologic malignancies [146,147] or genetic disorders [148–152]. Moreover,

recent studies demonstrate that treatment of genetic disorders by transplantation of autologous bone-marrow transduced with HIV derived LV vectors is feasible and effective in animals models [153]. Clinical trials to establish the safety and efficacy of this approach are underway.

Initially, the therapeutic application of stem cells in CF lung disease was expected to be as a tool to reconstitute airway epithelia damaged by bacterial infection and ongoing inflammation. The rationale is based on homing of transplanted stem cells to the damaged stem cell niche and their differentiation into epithelial cells. In the most promising clinical scenario, these cells should be obtained from the patient, engineered *ex-vivo* with wild-type *CFTR* gene-sequences (in a form suitable for providing life-long expression, such as an HAC or a LV vector), and reintroduced into the lung by local administration. This manoeuvre should itself not be inflammatory, and it is expected that an immune response should not be mounted. Thus, many issues have to be addressed before stem cell-based therapy could be a valid option for treatment of CF lung disease.

Systemic administration of bone marrow-derived stem cells in mice after total body irradiation and/or after treatment with epithelia-injuring reagents resulted in engraftment of stem cells mainly in alveolar spaces and sometimes in conducting airways (for review, see [154,155]). Considering that the therapeutic target in CF is the epithelium lining the conducting airways, proof-of-principle that intratracheal injection of stem cells into the lung gives rise to airway epithelial cells has been provided [156–159]. Nevertheless, the engraftment of bone marrow-derived stem/progenitor cells into the airways is a very inefficient process. Only a very small proportion (i.e. <0.01–0.025%) of lung epithelial cells, is derived from transplanted bone marrow-derived cells [154]. Although this could be due to technical problems, it might also be the case that whole bone marrow cells or selected populations have an inherent inability to engraft into the lung and differentiate into epithelial cells. The animal model employed and the type of damage to the epithelium might be another variable.

The source of stem cells may have important implications. Repopulation of injured airway epithelium *in vivo* has been attempted mostly with bone marrow-derived stem cells (for review, see [154,155]), but embryonic stem cells [158], cord blood-derived mesenchymal stem cells [160] and amniotic fluid stem cells [161] have also been used. However, the outcome in terms of transformation of stem cells into epithelial cells is similar to that obtained with bone marrow-derived cells. For example, a recent report on embryonic stem cells and cord blood-derived mesenchymal stem cells showed that 0.4 to 5.5% of stem cells engrafted in polidocanol-injured airways [158]. Moreover, the mouse might not be the most appropriate model to investigate this issue, due to anatomical and physiological differences compared to human lungs [94]. As discussed above (see Section 3. "Animal models old and new"), studies using large animal models are warranted.

Transduction of stem cells with retroviruses or lentiviruses in association with transplantation of engineered stem cells has been attempted in a few cases [158,160,162]. For example, Sueblinvong et al. [160] transduced human cord blood-derived mesenchymal stem cells with recombinant LV vectors expressing human CFTR. After systemic administration of the engineered cells to immunotolerant NOD-SCID mice, rare cells that had acquired cytokeratin and human CFTR expression were found in the airway epithelium [160]. Clearly, more studies are needed using appropriate animal models and stem cells with higher plasticity. However, concerns exist about the oncongenic potential of integrating LV vectors (see above, Section 2. "Viral and nonviral vectors"). These viruses belong to the same family as the oncoretroviruses, which are responsible for tumor initiation and propagation in patients treated with genetically manipulated allogeneic bone marrow cells (for review, see [163]).

Two groups have addressed whether transplanted stem cells restore CFTR function. Loi et al. [164] transplanted cultured bone marrow stromal cells expressing wild-type CFTR into transgenic CFTR knock-out mice. The authors observed the engraftment of donor-derived airway epithelial cells, but only in small numbers (approximately 0.025%). The total number of chimeric lung epithelial cells exhibiting CFTR expression was even less (0.01%). Bruscia et al. [165,166] transplanted CFTR-expressing bone marrow cells (obtained from green fluorescent protein transgenic mice) into irradiated CFTR knockout mice. Like Loi et al. [164], the authors observed very low levels of engraftment (0.01–0.1%) in the intestine, correlating with very low levels of CFTR mRNA expression. Surprisingly, the bioelectric profile of the CFTR knockout mice transplanted with CFTR-expressing bone marrow cells was significantly improved in both intestinal and nasal epithelia. Although the mechanism responsible for this effect is not known, these results imply that successful transplanation of very few cells is sufficient to restore CFTR function. *In vitro* studies suggest that expression of wild-type CFTR in only 6 to 20% of airway epithelia cells is sufficient to restore CFTR-mediated Cl^- secretion to airway epithelia [167,168]. However, *in vitro* and *in vivo* studies suggest that nearly every cell must express CFTR to reverse the defect in ENaC-mediated Na^+ absorption [167,169,170]. Clearly, the yield of transplanted stem cells, which become airway epithelial cells must be increased substantially to achieve a potentially therapeutic effect.

Concerning efficacy, engraftment of stem cells into the lung should have a curative effect on bacterial infection and lung inflammatory. So far only a preliminary report has been presented on this issue by van Heeckeren and colleagues (cited in [171]). When these authors transplanted wild-type bone marrow into a CFTR knockout mouse model of *P. aeruginosa* infection, they observed a 50% reduction of mortality. Interestingly, epithelial chimerism was not found since more than 97% of donor cells had a $CD45^+$ phenotype and were therefore not epithelial cells. This suggests that myeloid cells from bone marrow-derived stem cells control pulmonary inflammatory responses in this model of lung inflammation. Consistent with this idea, Bruscia et al. [172] recently demonstrated that macrophages derived from trans-

planted bone marrow participate in the hyperinflammatory response to *P. aeruginosa* endotoxin in CF mice. To what extent this applies to human CF lung disease remains to be established.

The issue of the pro-inflammatory or immunogenic potential of stem cell transplantation in the context of CF lung disease requires further study. Based on published literature, bone marrow mesenchymal stem cells should be a particular focus of study because they possess the potential to become airway epithelial cells, at least *in vitro* [173], and they promote tissue repair by secreting soluble factors that block apoptosis [174–176], inflammation [157,177–179], and other immune cell-mediated responses [180].

6. Concluding remarks

A plethora of delivery vectors have been evaluated for efficient and safe CFTR gene transfer to airway epithelia. However, there remains an absolute need to develop a vector suitable for repeated delivery that is capable of penetrating CF mucus. In parallel, the assessment of sensitive and specific surrogate biomarkers should be pursued.

The relevant cell compartment to target in CF airways still remains elusive. Bearing this caveat in mind, all epithelial cell types need be considered as potential candidates. Stem cells offer a unique opportunity to achieve this goal, but considering their limited tendency to differentiate into epithelial cells, their use for clinical application is still far from realistic. An alternative is now offered by the emergence of iPS cells as a new tool to obtain stem cells with pluripotent capacities from the same patient. iPS cells might be considered the ideal recipient for CFTR-expressing HACs engineered to carry all the necessary regulatory sequences for physiological expression of the *CFTR* gene.

Animal models will continue to be important tools for the development and assessment of gene and cell therapies. Novel methods for creating mutations in species other than mice offer the imminent prospect of accurate models of CF lung disease. Time will tell if the pig model already fulfils this goal. Finally, the success or failure of any curative postnatal therapy for CF, including gene therapy, will also determine the future relevance of *in utero* gene therapy for this still incurable and severely life-shortening disease.

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